Safety and Immunogenicity Trial in Adult Volunteers of a Human Papillomavirus 16 L1 Virus-Like Particle Vaccine

Clayton D. Harro, Yuk-Ying Susana Pang, Richard B. S. Roden, Allan Hildesheim, Zhaohui Wang, Mary Jane Reynolds, T. Christopher Mast, Robin Robinson, Brian R. Murphy, Ruth A. Karron, Joakim Dillner, John T. Schiller, Douglas R. Lowy

Background: **Studies in animal models have shown that systemic immunization with a papillomavirus virus-like particle (VLP) vaccine composed of L1, a major structural viral protein, can confer protection against subsequent experimental challenge with the homologous virus. Here we report results of a double-blind, placebo-controlled, dose-escalation trial to evaluate the safety and immunogenicity of a human papillomavirus (HPV) type 16 (HPV16) L1 VLP vaccine in healthy adults.** ***Methods:*** **Volunteers were given intramuscular injections with placebo or with 10- or 50-μg doses of HPV16 L1 VLP vaccine given without adjuvant or with alum or MF59 as adjuvants at 0, 1, and 4 months. All vaccine recipients were monitored for clinical signs and symptoms for 7 days after each inoculation. Immune responses were measured by an HPV16 L1 VLP-based enzyme-linked immunosorbent assay (ELISA) and by an HPV16 pseudovirion neutralization assay. The antibody titers were given as the reciprocals of the highest dilution showing positive reactivity in each assay. All statistical tests were two-sided.** ***Results:*** **The prevaccination geometric mean ELISA titer for six seropositive individuals was 202 (range, 40–640). All vaccine formulations were well tolerated, and all subjects receiving vaccine seroconverted. Serum antibody responses at 1 month after the third injection were dose dependent in recipients of vaccine without adjuvant or with MF59 but were similar at both doses when alum was the adjuvant. With the higher dose, the geometric means of serum ELISA antibody titers (95% confidence intervals) to purified VLP 1 month after the third injection were as follows: 10 240 (1499 to 69 938) without adjuvant, 10 240 (1114 to 94 145) with MF59, and 2190 (838 to 5723) with alum. Responses of subjects within each group were similar. Neutralizing and ELISA antibody titers were highly correlated (Spearman correlation = .85), confirming that ELISA titers are valid proxies for neutralizing antibodies.** ***Conclusions:*** **The HPV16 L1 VLP vaccine is well tolerated and is highly immunogenic even without adjuvant, with the majority of the recipients achieving serum antibody titers that were approximately 40-fold higher than what is observed in natural infection. [J Natl Cancer Inst 2001;93: 284–92]**

Invasive cervical cancer develops in approximately 400 000 women per year worldwide and results in approximately 200 000 deaths per year *(1,2).* The greatest burden of disease is in developing countries, where cervical cancer is often the most frequent female malignancy, and may constitute up to one quarter of all female cancers. Early detection of premalignant cervical neoplasia is possible with Pap smears, but it has been difficult to establish screening programs in developing countries. In the United States, Pap smear screening and follow-up have been estimated to cost more than 5 billion dollars annually, but the widespread availability of such screening has been associated with a 75% reduction in the incidence of cervical cancer *(3).* However, in the United States, this cancer still accounts for about 7% of all female malignancies and about 5000 deaths per year *(4).*

Clinical and molecular epidemiologic investigations have identified human papillomavirus (HPV) as the major cause of cervical cancer and cervical dysplasia *(5,6).* Virtually all cervical cancers contain the genes of high-risk HPVs (most commonly, types 16, 18, 31, and 45), and the relative frequency with which these types are found is remarkably similar in most regions of the world *(6,7).* HPV16 is found in approximately 50% of cervical cancers, and types 18, 31, and 45 account for an additional 25%–30% of HPV-positive tumors.

Identification of HPV as a causal factor in virtually all cervical cancers implies that development of an effective vaccine against high-risk HPV could prevent the premalignant and malignant disease associated with HPV infection. Since prophylactic viral vaccines have a long record as a cost-effective approach to prevent infection or modify disease, such a vaccine might also lower the cost of screening and treating premalignant cervical disease. HPVs are DNA tumor viruses that contain oncogenes. There might be theoretic arguments against the presence of such genes, which can disrupt normal growth controls, in a vaccine destined for normal individuals. To develop a prophylactic vaccine against HPV infection, we and others *(8)* have, therefore, taken a subunit vaccine approach, analogous to that

Affiliations of authors: C. D. Harro, M. J. Reynolds, T. C. Mast, R. A. Karron (Center for Immunization Research, Department of International Medicine, School of Hygiene and Public Health), R. B. S. Roden (Department of Pathology, School of Medicine), The Johns Hopkins University, Baltimore, MD; Y.-Y. S. Pang, J. T. Schiller, D. R. Lowy (Laboratory of Cellular Oncology, Division of Basic Sciences), A. Hildesheim (Environmental Epidemiology Branch, Division of Cancer Epidemiology and Genetics), National Cancer Institute, Bethesda, MD; Z. Wang, J. Dillner, Microbiology and Tumor Biology Centre, Karolinska Institute, Stockholm, Sweden; R. Robinson, Novavax, Inc., Columbia, MD; B. R. Murphy, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda.

Correspondence to: Douglas R. Lowy, M.D., National Institutes of Health, Bldg. 36, Rm. 1D-32, Bethesda, MD 20892 (e-mail: drl@helix.nih.gov).

See "Notes" following "References."

used successfully against hepatitis B-induced disease, including hepatocarcinoma *(9)*.

Papillomaviruses encode a major capsid protein, L1, that has the intrinsic capacity to self-assemble into virus-like particles (VLPs) in the absence of other viral gene products *(10–13)*. Recombinant L1 VLPs are morphologically indistinguishable from authentic virions, contain the immunodominant conformationally dependent neutralization epitopes present in authentic virions, and have the ability to generate high titers of type-specific neutralizing antibodies *(8)*.

Several trials of preventive papillomavirus vaccine candidates using L1 VLPs purified from insect cells have been conducted by use of the cutaneous cottontail rabbit papillomavirus (CRPV), the oral mucosal bovine papillomavirus 4 (BPV4), or the canine oral papillomavirus (COPV) disease model in its natural host. Three subcutaneous injections of CRPV L1 VLPs given without adjuvant, or combined with alum or Freund's adjuvant, protected rabbits against persistent infection and subsequent carcinoma after high-dose CRPV challenge *(14,15)*. Protection lasted at least 1 year *(15)*. Similarly, dogs or calves given two intramuscular injections of COPV L1 VLPs (without adjuvant) or BPV4 L1 VLPs (with alum), respectively, were protected from subsequent oral mucosal challenge *(16,17)*. In the CRPV and COPV models, passive transfer of serum or immunoglobulin (Ig) G from animals immunized with the L1 VLPs protected naive animals challenged with the homologous virus, indicating that neutralizing antibodies were sufficient to confer protection *(14,16)*.

Since papillomaviruses are species specific and HPVs do not induce disease in animals *(4),* further HPV vaccine development and evaluation require studies in humans. Therefore, as an initial step in demonstrating proof-of-principle, we developed a prophylactic recombinant HPV16 L1 VLP vaccine candidate, produced in insect cells with recombinant baculovirus, for testing in humans. This study was designed to evaluate the safety and immunogenicity in healthy young adults of two dose levels (10 and 50 μg) of the HPV16 L1 VLP vaccine given in aqueous solution without adjuvant or mixed with alum or MF59 adjuvant.

Subjects and Methods

Vaccine

Recombinant baculovirus expressing the full-length L1 capsid gene of HPV16 strain 114K *(11)* as VLPs was constructed by use of the bacmid system *(18)*. A 1.5-kilobase *Bgl*II DNA fragment from pEVnod-KL1 *(11)* containing the HPV16 L1 gene was cloned in the *Bam*HI site downstream of the polyhedrin promoter within the polh locus in the baculovirus donor plasmid pFASTBAC-1 (Life Technologies, Inc. [GIBCO BRL], Rockville, MD) by site-specific recombination in *Escherichia coli* DH10Bac1. Recombinant baculovirus containing the HPV16 L1 DNA was isolated from Sf-9 insect cells transfected with the recombinant bacmid DNA by use of the cationic lipid Cellfectin (Life Technologies, Inc.). Recombinant baculovirus was plaque purified three times and screened for insert integrity by DNA sequencing and L1 capsid antigen expression in insect cells by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analyses. One recombinant baculovirus isolate that expressed high levels of L1 protein intracellularly and extracellularly was amplified in Sf-9 cells, tested for microbial contaminants and adventitious agents, and designated the master virus seed stock (bHPV16 L1 R-212). Working virus seed stocks of bHPV16 L1 virus were produced in Sf-9 cells infected for 3 days at a multiplicity of infection (moi) of 0.01 plaque-forming unit (pfu)/cell.

Production of clinical lots of recombinant HPV16 L1 VLP vaccines was performed in accordance to good manufacturing practice guidelines for well-characterized biologicals at the Vaccine Production Facility of Novavax, Inc. (formerly DynCorp) in Columbia, MD. Production lots of recombinant HPV16 L1 VLPs were manufactured in Sf-9 cells (2–3 × 10^6 cells/mL in 16.8-L-size batches) infected with bHPV-16 L1 virus at an moi of 3–5 pfu's/cell for 4–5 days at 28 °C as described previously *(19)*. Sf-9 cells were cultivated as suspension cultures by use of Sf-900 II serum-free medium (Life Technologies, Inc.). Extracellular HPV16 L1 VLPs were recovered from infected cell suspension by low-speed centrifugation (1000*g* for 10 minutes at 4 °C), and supernatants were clarified by centrifugation (10 000*g* for 30 minutes at 4 °C). Clarified supernatants were concentrated 15-fold by ultrafiltration with the use of hollow fiber membranes (UFP-5-C-6A, MWCO 500 000) (A/G Technologies) and diafiltered against 20 volumes of phosphate-buffered saline (PBS) (pH 7.2). The dialysate was clarified by centrifugation as described above, the clarified supernatant was loaded onto 30% sucrose cushions in PBS, and the recombinant VLPs and baculovirus were pelleted through the sucrose cushions by ultracentrifugation (26 000*g* for 3 hours at 4 °C) by use of swinging bucket rotors. Pellets were solubilized in PBS and loaded onto 25%–65% sucrose step gradients. VLPs were resolved as bluish bands on gradients by ultracentrifugation (25 000*g* for 1 hour at 4 °C) by use of swinging bucket rotors. Successive rounds of ultracentrifugation on sucrose gradients were used to obtain VLPs with a purity of more than 95%. VLPs were recovered from banded VLPs by ultracentrifugation (26 000*g* for 3 hours at 4 °C). Sucrose was removed from pelleted VLPs by dialysis against PBS. The VLPs were then diluted to 0.3 mg/mL and filtered aseptically through 0.22-μm membranes. The filtered VLPs were designated the final bulk product and stored at −20 °C. The final bulk product underwent both safety and analytic testing, and the batch records were audited by quality assurance before release of the final bulk product for formulation of final container vaccine product. The integrity of the VLPs was monitored by their morphology in the electron microscope, their ability to hemagglutinate mouse red blood cells efficiently *(20),* and their strong reactivity to HPV16 conformational and neutralizing monoclonal antibodies (H16.V5, H16.E70, and H16.U4) *(21,22)* and nonreactivity to an HPV11 neutralizing monoclonal antibody (H11.B2) *(23)*.

The final bulk product of recombinant HPV16 L1 VLPs was formulated in a final volume of 0.5 mL as a nonadjuvanted vaccine at a 10- and 50-μg dose or in a final volume of 0.25 mL to be mixed with MF-59 adjuvant (Chiron Corporation, Emeryville, CA), which is a microfluidized oil-in-water emulsion consisting of 1.25 mg of sorbitan amonoleate (Tween 80®), 1.25 mg of sorbitan trioleate (Span 85®), and 10.75 mg of squalene per 0.5-mL dose. At the time of vaccine administration, 0.25 mL of MF59 emulsion was combined with 0.25 mL of vaccine to yield a vaccine dose of 10 and 50 μg. Alternatively, the final bulk product was formulated with alum (aluminum potassium sulfate, 10%; EM Merck, Darmstadt, Germany) at 120 mg of alum per mg of VLP antigen. The final volume of the VLP–alum vaccine was 0.5 mL for 10- and 50-μg doses. Sterile saline served as the placebo vaccine. Formulated VLPs were dispensed aseptically into sterile vials (3.0-mL size, type 1 borosilicate glass, silanized, depyrogenated; Wheaton Glass, Wheaton, MD) as a single-unit dose and were designated final container vials. Vials containing only VLP antigen were stored at −20 °C before administration, whereas vials containing VLP antigen adsorbed to alum were stored at 4 °C because of stability concerns.

Study Design

This double-blind, randomized, placebo-controlled, phase I safety and immunogenicity trial was conducted at The Johns Hopkins University Center for Immunization Research (Baltimore, MD). Guidelines for human experimentation of the Joint Committee for Clinical Investigation of The Johns Hopkins University School of Medicine and its institutional review board were followed in the conduct of this study. Seventy-two healthy, human immunodeficiency virus (HIV)-1-seronegative, 18- to 29-year-old volunteers (58 females and 14 males) were recruited for this study. Subjects were determined by history to be at low risk for HPV16 exposure. Individuals were not eligible to participate if they had a history of more than four lifetime sexual partners or more than two sexual partners within the preceding 6 months. Additional exclusion criteria included history of abnormal cervical cytology, immunodeficiency, anaphylaxis to medicines or vaccines, receipt of blood products within 3 months of enrollment, current pregnancy or lactation, and any other condition that might interfere with the study objectives. All aspects of the protocol were explained to the subjects who met the eligibility criteria, and informed, witnessed, written consent was obtained. Preimmune HPV16 serostatus was not a criterion for eligibility, since it was expected that only about 10% of the volunteers would be seropositve (in this study, only six of 72 volunteers were found to be seropositive at study entry), its omission simplified recruitment, enrollment, and initiation of vaccination, and the presence of a few seropositive vaccine recipients would enable us to monitor the response of such individuals.

Before enrollment, a medical history was obtained from each subject, a physical examination was performed, and the following laboratory tests were done (Quest Diagnostics; Baltimore, MD): complete blood cell count (CBC), platelet count, alanine aminotransferase (ALT), serum creatinine, hepatitis B surface antigen, HIV antibody test, and urine dipstick for hemoglobin and protein. Volunteers were eligible to enroll if their medical history, physical examination, and laboratory tests were without clinically significant abnormalities. Individuals with clinically significant abnormalities were not enrolled but were counseled and referred for medical consultation as appropriate. To qualify for enrollment and subsequent vaccination, female subjects were required to have a negative urine pregnancy test on the day of each vaccination and to use an acceptable method of birth control until completion of the study.

To determine whether the dose of HPV16 L1 VLP vaccine and/or the addition of alum or MF59 adjuvant would influence the reactogenicity or immune response, the trial was conducted in a dose-escalation manner, starting with 10 μg of HPV16 L1 VLP vaccine given alone, with alum, or with MF59 adjuvants. When this dose was determined to be safe, we then evaluated a 50-μg dose of HPV16 L1 VLP vaccine given alone, with alum, or with MF59 adjuvant (Table 1). For each dose/adjuvant group, 10 subjects were randomly assigned to receive investigational vaccine, and two were randomly assigned to receive placebo (sterile physiologic saline). Subjects received vaccine or placebo as an intramuscular injection (0.5 mL) in the deltoid region at months 0, 1, and 4. To maintain blinding, the individual who prepared and administered the vaccine was not involved in data collection or clinical evaluation of volunteers.

Subjects were evaluated clinically, and blood was collected for clinical and immunologic tests before each injection (months 0, 1, and 4) and 1 month after each injection. Clinical laboratory tests done at each of these visits included the following: CBC, platelet count, serum ALT, serum creatinine, urine protein, and urine hemoglobin measurements. After each injection, oral temperatures were recorded after 30 minutes, 6 hours, and daily for 6 days. Adverse reactions were monitored by study staff in the clinic at 30 minutes and 2 days after each injection and by telephone for the following 6 days. Clinical signs and symptoms were scored as follows: none = no reaction; mild = transient or mild discomfort; moderate = mild to moderate limitation in activity; and severe = marked limitation in activity.

Serologic Assays

IgG-specific HPV16 L1 VLP-based enzyme-linked immunosorbent assays (ELISAs) were performed in a 96-well plate format as described previously *(24),* except that 200 ng of VLPs was used per well and end point dilution titers were determined. VLPs for the ELISAs were purified from the nuclei of HPV16 L1 recombinant baculovirus-infected Sf-9 cells as described previously *(11).* Fourfold dilutions of each serum were assayed, starting at a dilution of 10. Sera were designated ELISA positive at a given dilution if the absolute optical density (OD) was greater than or equal to 0.2 and was at least double the reactivity of the same serum dilution in a well containing blocking buffer but no VLPs. Seroconversion was defined as a fourfold or greater rise in titer. Vaccine recipients were considered to be HPV16 seropositive at enrollment if their prevaccination serum demonstrated an ELISA antibody titer that was greater than or equal to the reactivity of a standard pooled serum, which was assayed on the same plate (generally OD of 0.4–0.6 at 1 : 40 dilution). Reactivity to the standard serum had been validated as a cut point for seropositivity in a previous seroepidemiologic study *(24).*

Table 1. Immunization scheme for safety and immunogenicity trial of human papillomavirus 16 L1 virus-like particle vaccine with or without alum or MF59 adjuvant*

Study part	No. of volunteers	Vaccine dose	Adjuvant
I	10	10 μg	—
	2	Placebo	—
	10	10 μg	0.5 mg alum
	2	Placebo	—
	10	10 μg	0.25 mL MF59
	2	Placebo	—
II	10	50 μg	—
	2	Placebo	—
	10	50 μg	0.5 mg alum
	2	Placebo	—
	10	50 μg	0.25 mL MF59
	2	Placebo	—

*All vaccinations were given intramusculary in 0.5 mL of inoculum at 0, 1, and 4 months. Placebo recipients received 0.5 mL of saline.

The detailed procedure for IgM, IgA, and IgG isotyping and for determining IgG subclass with subclass-specific second antibodies is reported elsewhere *(25).* Sera from the individuals receiving vaccine without adjuvant or with alum were tested in triplicate at a dilution of 1 : 50 for IgG and IgG1 or at a dilution of 1 : 20 for IgG2, IgG3, and IgG4 by use of the corresponding isotype- or subclass-specific second antibody. A sample was considered to be positive if, after subtraction of the reactivity to denatured BPV1 VLPs, the OD was greater than the mean OD plus three standard deviations from the mean for a panel of sera from virgin women and was at least 0.100.

In vitro HPV16 pseudovirion neutralization assays were performed as described previously *(26).* Briefly, infectious virions composed of the HPV16 L1 and L2 capsid proteins and the BPV1 genome were generated by infection of BPHE-1 hamster cells (which contain autonomously replicating BPV genomes) with replication-defective Semliki Forest Virus vectors expressing HPV16 L1 and L2 genes. Individual infections by the pseudotype virions were detected as transformed foci in a monolayer of mouse C127 cells. Fourfold dilutions of the sera, starting with a dilution of 1 : 10, were mixed with approximately 50–100 focus-forming units of pseudotype virions and assayed for inhibition of focal transformation. Neutralization was defined as at least a 50% reduction in the number of foci compared with the number obtained in the absence of human serum. The ELISA and neutralizing antibody titers are given as the reciprocal of the highest positive dilution for each assay.

Statistical Methods

The data for local and systemic reactions were grouped by vaccine dose and adjuvant. Data from placebo recipients were pooled for analysis. We compared proportions of reactions among different categories of volunteers by use of a continuity corrected test for proportions between two independent groups *(27).* Nonparametric statistics (Kruskal–Wallis test) were used to make comparisons across groups at a given time. The Kruskal–Wallis test was also used to compare the distribution of antibody titers in different groups over time *(28).* The Spearman correlation coefficient was used when comparing ELISA results against those obtained by using the neutralization test *(28).* Categorized ELISA and neutralization results were compared with the use of the Kappa statistic, and overall agreement percentages were also computed *(29).* All statistical tests were two-sided.

RESULTS

This was a double-blind, randomized, placebo-controlled safety and immunogenicity dose escalation trial of HPV16 L1 VLPs that were made in insect cells with a recombinant baculovirus. An electron micrograph of the VLP preparation is given in Fig. 1, A. When the VLP preparation was denatured and subjected to gel electrophoresis, L1 was the only band seen in the gel (Fig. 1, B). The trial tested two vaccine doses, 10 and 50 μg, without adjuvant or with alum or MF59 adjuvant (Table 1). Each vaccine preparation was given at 0, 1, and 4 months. The demographic characteristics of the study population are shown in Table 2. In the first part of the study, 35 of the 36 subjects were randomly assigned and received three injections of either 10 μg of HPV16 L1 VLP vaccine (with or without adjuvant) or placebo. One placebo recipient moved away before receiving the third injection. In the second part of the study, 33 of the 36 subjects were randomly assigned and received three injections of either 50 μg of HPV16 L1 VLP vaccine (with or without adjuvant) or placebo. Two subjects who did not complete the scheduled series of injections were in the group that received 50 μg of HPV16 L1 VLP with alum. One of these subjects received two immunizations but did not return for follow-up visits thereafter. The other subject received the first two immunizations, but the third was not given because of 1+ hemoglobin in the urine (as

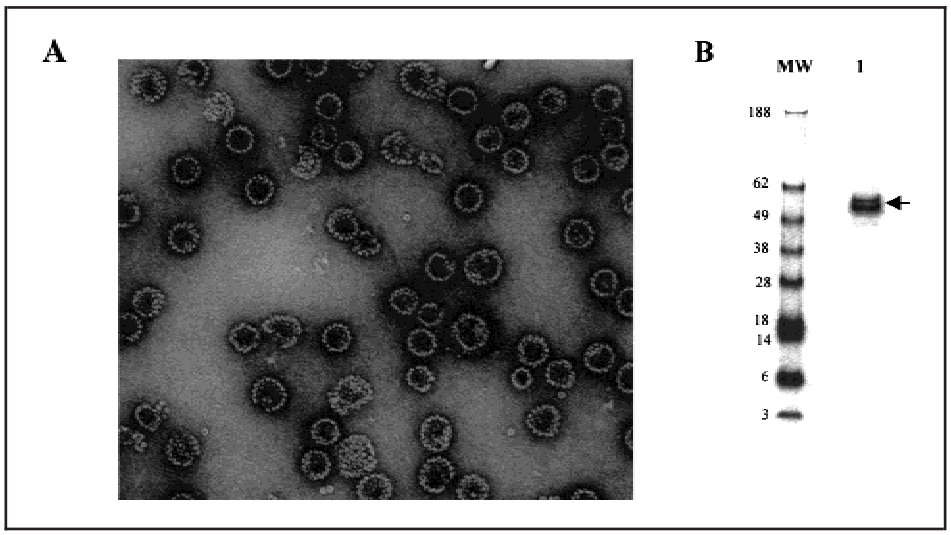

Fig. 1. Human papillomavirus type 16 L1 virus-like particles (HPV16 L1 VLPs) used as vaccine in the trial. **A**) transmission electron micrograph showing the morphology of the HPV16 L1 VLPs in the vaccine preparation (original magnification ×36 000). **B**) Coomassie Blue-stained gradient polyacrylamide gel electrophoresis of the vaccine preparation showing L1 as the only detectable band. MW = molecular weight.

Table 2. Demographics of study population by human papillomavirus type 16 L1 virus-like particle (HPV16 L1 VLP) vaccine dose group*

Demographic	HPV16 L1 VLP, 10 μg	HPV16 L1 VLP, 50 μg
Sex		
Female	30	28
Male	6	8
Race		
Caucasian	28	26
Asian	6	7
African-American	2	3
Mean age, y	24	24
Mean No. of sexual partners		
Lifetime	2	2
6 mo. before study entry	0.7	0.8

*Includes concurrent placebo recipients.

determined by standard dipstick measurement, calibrated as 0 [neg]–4+) noted on the day the third vaccination was scheduled. Subsequent urologic evaluation of this volunteer revealed no abnormality. The third subject who did not complete the full series of injections was a placebo recipient who was lost to follow-up after the second injection.

Clinical Responses

Administration of three injections of HPV16 L1 VLP vaccine, given alone or with alum or MF59 adjuvant, was well tolerated at both dose levels (Table 3). For each group, the frequency and severity of reactions following each of the three doses were similar, and reactions for all three vaccinations have, therefore, been grouped together. Most of the local and systemic reactions were classified as mild. Clinical responses of the subjects who received 10 μg of vaccine without adjuvant were

Table 3. Cumulative percent reactions in subjects within 1 week of vaccination*

	Vaccine or placebo administered†						
	HPV16 L1 VLP, 10 μg			HPV16 L1 VLP, 50 μg			
Reaction	Aqueous, no adjuvant (30)	Alum (29)	MF59 (30)	Aqueous, no adjuvant (30)	Alum (28)	MF59 (30)	Placebo, saline (35)
Local							
Pain (any)	23.3	36.7	80.0	56.7	51.9	93.3	22.9
Mild‡	23.3	33.3	66.7	56.7	40.7	60.0	22.9
Moderate‡	0.0	3.3	13.3	0.0	11.1	33.3	0.0
Erythema§	3.3	6.7	3.3	0.0	7.4	16.7	2.9
Induration§	3.3	3.3	3.3	6.7	3.7	16.7	2.9
Systemic							
Fever >100 °F	0.0	0.0	3.3	0.0	0.0	0.0	2.9
Headache	6.7	10.0	16.7	10.0	11.1	16.7	5.7
Mild‡	3.3	10.0	3.3	10.0	7.4	13.3	2.9
Moderate‡	3.3	0.0	13.3	0.0	3.7	3.3	2.9
Nausea	0.0	3.3	3.3	3.3	0.0	6.7	2.9
Malaise	3.3	3.3	3.3	10.0	0.0	0.0	2.9
Myalgia	0.0	0.0	3.3	10.0	0.0	0.0	2.9

*If an individual had any reaction, e.g., pain, following a particular injection, it was recorded as one episode of that reaction, regardless of its duration (e.g., mild pain lasted <48–72 hours). The percent numbers in the substrata for pain and headache do not necessarily add up to total percent numbers with those reactions due to rounding. HPV16 L1 VLP = human papillomavirus type 16 L1 virus-like particle.

†Numbers in parentheses indicate the total number of inoculations in the group. Numbers in columns indicate percent of total inoculations with this reaction.

‡Maximum reported pain or headache. All reactions were classified as mild or moderate (no severe reactions were reported).

§Erythema or induration >5 mm in diameter.

almost identical to those of the placebo group. Subjects who received 50 μg of vaccine without adjuvant reported local side effects about twice as frequently as the placebo recipients. Recipients of either 10 or 50 μg of vaccine with MF59 reported pain at the injection site more frequently than recipients of vaccine with alum or without adjuvant (10 μg—24 (80%) of 30 versus 11 (36.7%) of 30 and seven (23.3%) of 30; $P = .002$ and $P = .0001$, respectively; 50 μg—28 (93.3%) of 30 versus 14 (51.9%) of 27 and 17 (56.7%) of 30; $P = .0005$ and $P = .003$, respectively). However, pain was mild to moderate in intensity and resolved spontaneously within 48–72 hours in all subjects. Similarly, recipients of 50 μg of vaccine with MF59 reported local induration and/or erythema more frequently than recipients of 50 μg of vaccine with alum or without adjuvant. Of note, one recipient of 50 μg of vaccine with MF59 reported erythema that peaked at 60 mm in diameter on the day of the third vaccination and resolved on day 2 after vaccination. A second recipient of 50 μg of vaccine with MF59 reported 45 mm of erythema and induration that began on the day of the second vaccination and resolved on day 4 after vaccination. Neither of these subjects required medication or other clinical intervention. There was no notable difference in the clinical responses of the five vaccine recipients who were seropositive at entry compared with the seronegative vaccine recipients in the same group nor was the frequency or severity of reactions greater after the third vaccination than after the initial vaccinations.

Two subjects had clinically significant laboratory abnormalities. As described above, one subject in the group that received 50 μg of vaccine with alum had transient idiopathic microscopic hematuria. A second subject was noted to have asymptomatic ALT elevation 1 month after receiving the second vaccine dose (ALT = 356 U; normal range, 0–48 U), which resolved during the subsequent month. Clinical evaluation, including laboratory studies for hepatitis, was unrevealing, except that the subject recalled a similar asymptomatic episode (not reported before enrollment in the vaccine study) of ALT elevation that was discovered 2 years earlier after donating blood. The clinical evaluation at that time was also negative. After resolution of the ALT abnormality, the subject received the third vaccine dose and had no further elevation in the ALT level. Clinical laboratory results for all other subjects were unremarkable.

HPV16-Specific Antibody Responses

The volunteers were evaluated for serum IgG responses to the vaccine by use of an HPV16 VLP ELISA *(24)* (Table 4). Overall, the sexual-history screening tool used in this study was an effective predictor of baseline HPV16 seronegativity by ELISA. A total of six of 72 subjects were IgG seropositive by ELISA at study entry. The geometric mean ELISA titer (GMT) of the prevaccination sera from these six individuals was 202 (range, 40–640). Four of these subjects received 10 μg of HPV16 L1 VLP vaccine (one without adjuvant, two with alum, and one with MF59), one received 50 μg of HPV16 L1 VLP vaccine without adjuvant, and one received placebo. The final serum ELISA titers for each of the five vaccine recipients who were seropositive before vaccination were no more than one fourfold dilution above the final serum GMT of the prevaccination seronegative vaccinees in the same group.

Excluding the initial seropositive subjects, GMT ELISA titers at entry were less than or equal to 20 for each group, and there was no statistically significant difference in baseline antibody titers between groups ($P = .40$) (Table 4). None of the placebo recipients seroconverted. By contrast, all vaccine recipients seroconverted within 1 month after the second vaccination (data shown only as group mean). In all groups receiving investigational vaccine, the titers 3 months after the second injection (month 4) waned compared with those achieved 1 month after this injection (month 2) (P values for all comparisons = >.1, with the exception of the comparison for the group that received 10 μg of vaccine with MF59, where $P = .08$). Conversely, a clear boost in the titers was seen 1 month after the third injection (month 5) (P values ranging from .002 to .04), except for the group that received 50 μg of VLP in alum, whose titers showed a small increase ($P = .26$).

In each group that received vaccine (alone or with adjuvant), peak titers were observed at month 5 (1 month after the third

Table 4. Geometric mean titers for human papillomavirus type 16 (HPV16)-specific antibodies by enzyme-linked immunosorbent assay (ELISA) and neutralization assay

HPV16 L1 virus-like particle vaccine dose—adjuvant (No.*)	ELISA titers (95% confidence interval)						Neutralization titers, month 5
	Month						
	0†	1†	2	4†	5	P‡	
10 μg—no adjuvant (9)	13 (4 to 43)	54 (16 to 179)	403 (27 to 6100)	159 (11 to 2407)	640 (164 to 2490)	.0001	nt§
10 μg—alum (8)	12 (3 to 51)	67 (6 to 807)	639 (82 to 4983)	380 (50 to 2869)	3040 (315 to 29 352)	.0001	nt
10 μg—MF59 (9)	18 (4 to 75)	137 (55 to 339)	1880 (195 to 18 094)	746 (89 to 6240)	3480 (179 to 67 811)	.0001	nt
50 μg—no adjuvant (9)	19 (4 to 100)	470 (24 to 9158)	5530 (503 to 60 747)	2560 (375 to 17 484)	10240 (1499 to 69 938)	.0001	560
50 μg—alum (10)	10 (2 to 45)	211 (38 to 1177)	2190 (885 to 5417)	1520 (372 to 6203)	2190‖ (838 to 5723)	.0001	86
50 μg—MF59 (10)	16 (3 to 77)	735 (37 to 14 619)	2560 (278 to 23 536)	1690 (270 to 10 577)	10240 (1114 to 94 145)	.0001	840
Placebo (11)	11 (3 to 42)	17 (3 to 83)	17 (3 to 83)	18 (4 to 92)	21 (4 to 107)	.43	<10
Kruskal–Wallis P value¶	.40	.0001	.0001	.0001	.0001		
Kruskal–Wallis P value, excluding placebo group¶	.44	.0001	.0004	.0003	.0001		

*Number of subjects per group, excluding individuals who were ELISA positive at entry.

†Time of vaccination.

‡Results comparing geometric mean titers at different time points for a given arm.

§nt = not tested.

‖Excludes results for two subjects who did not receive their third vaccination dose.

¶Results comparing geometric mean titers between arms for a given time point.

injection). The month-5 antibody titers for the groups that received 10 μg of vaccine with alum and 10 μg of vaccine with MF59 were similar ($P = .76$) and were significantly higher ($P = .007$ and $P = .01$, respectively) than the month 5 antibody titers of the group that received 10 μg of HPV16 L1 VLP alone. Thus, within the low-dose group, the addition of alum or MF59 adjuvant enhanced the immune response.

Compared with the month-5 titers at the 10-μg dose, titers for the 50-μg groups were markedly higher in recipients of vaccine without adjuvant, modestly higher in recipients of vaccine with MF59, and slightly lower in recipients of vaccine with alum. In contrast to the results obtained with the 10-μg vaccine dose, the ELISA antibody titers achieved in the group that received 50 μg of vaccine without adjuvant were equivalent to those in the group that received 50 μg of vaccine with MF59 ($P = .58$). Month-5 antibody titers in these two groups were significantly higher ($P = .002$ and $P = .001$, respectively) than the antibody titers in the group that received vaccine with alum.

The sera from subjects vaccinated without adjuvant or with alum were also analyzed in HPV16 VLP ELISAs specific for IgM and IgA and for IgG subclasses (Table 5). The majority of the antibody response to VLP vaccination was of the IgG1 subclass. As expected, most vaccine recipients became transiently seropositive for HPV16 IgM antibodies, which were usually detected in the sample taken 1 month after the initial vaccination. Most vaccine recipients also became seropositive for serum IgA; however, relative to IgG responses, the responses varied considerably and were generally weaker. All vaccine recipients became strongly seropositive for IgG1, and the responses closely paralleled the response to total IgG. In contrast, only one subject became weakly, although consistently, seropositive in the IgG2 assay. Responses to IgG3 and IgG4 were weak and variable.

Table 5. Number of vaccine recipients who became ELISA seropositive for human papillomavirus type 16 virus-like particle-specific immunoglobulin (Ig) isotype or subclass*

Group (No.)†	IgM	IgA	IgG	IgG1	IgG2	IgG3	IgG4
10 μg of vaccine without adjuvant (9)	8	3	9	9	0	5	2
10 μg of vaccine with alum (8)	5	7	8	8	1	1	2
50 μg of vaccine without adjuvant (9)	8	8	9	9	0	3	1
50 μg of vaccine with alum (8)	7	7	8	8	0	2	0

*Control subjects receiving placebo and those subjects who were IgG seropositive at entry were excluded. ELISA = enzyme-linked immunosorbent assay.
†Number in parentheses indicates the number of subjects per group.

The VLP ELISAs may measure a combination of neutralizing and non-neutralizing HPV virion antibodies, as well as antibodies to immunogenic insect cell or baculovirus proteins common to the vaccine and ELISA antigen. Therefore, it was important to determine directly whether the HPV L1 VLP vaccine was able to induce virion neutralizing antibodies, which are expected to correlate most closely with the potential for protection. For this analysis, we employed an HPV16 pseudovirion neutralization assay developed previously *(26).* On the basis of studies with animals, this assay is approximately 20- to 30-fold less sensitive than the HPV16 ELISA *(30).* For analysis of the current trial, month 5 sera from recipients of the 50-μg dose (with or without adjuvant) were studied with the neutralization assay (Table 4; Fig. 2).

As expected, the neutralization titers were much lower than those obtained with the ELISA (Fig. 2). However, sera from all vaccine recipients had detectable neutralizing antibodies. In contrast, none of the placebo recipients developed a detectable neutralizing antibody response (data not shown). There was a remarkable consistency of neutralizing titer within each vaccine/adjuvant group. With the exception of one individual in the MF59 group with an unusually strong response, neutralizing titers varied by no more than one fourfold dilution from the median (Fig. 2). In addition, the neutralizing antibody titers were highly correlated with ELISA antibody titers (Spearman correlation = .85). Similarly, when neutralization assay and ELISA

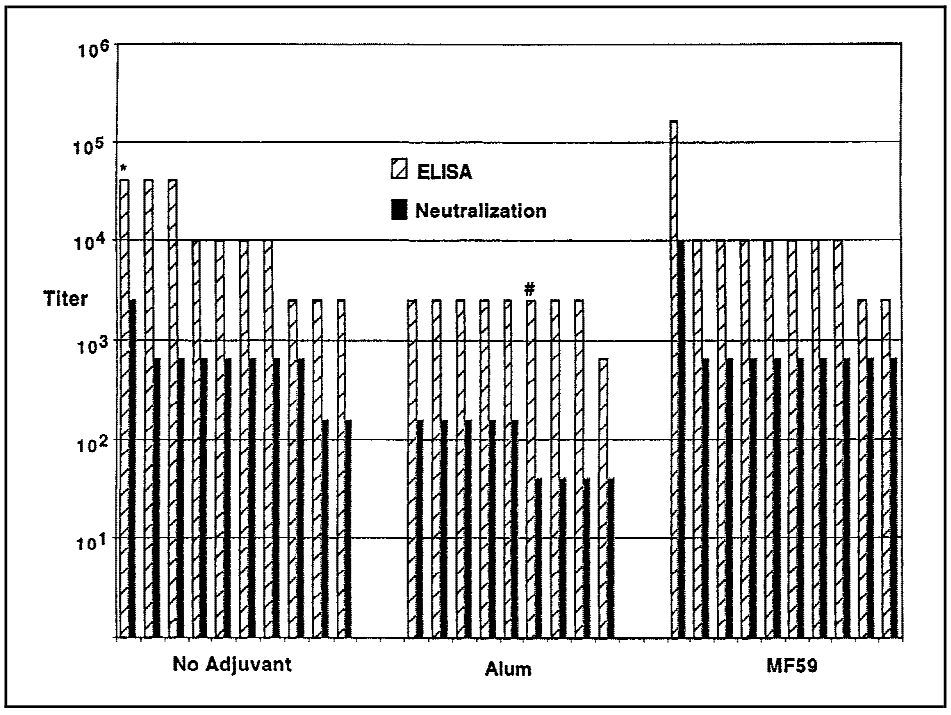

Fig. 2. Month-5 serum enzyme-linked immunosorbent assay (ELISA) titers and neutralization antibody titers for individuals vaccinated with three doses of 50 μg of human papillomavirus type 16 L1 virus-like particles (HPV16 L1 VLPs). HPV16 L1 VLP ELISA titers are indicated by the **hatched bars,** and HPV16 pseudovirion neutralizing titers are indicated by the **black bars.** The individuals vaccinated without adjuvant, with alum, and with MF59 are grouped from left to right. * indicates an individual whose preinoculation serum was ELISA positive. # indicates an individual who received only the first two immunizations.

results were categorized as low, medium, or high titers (ELISA—low = ≤160, medium = 640–2450, and high = >2450; neutralization assay—low = ≤10, medium = 40–160, and high = >160) and compared, the kappa coefficient was .80 (95% confidence interval = 0.60 to 0.99) and the percent agreement was 87.5%. These data suggest that ELISA titers were effective surrogates for neutralizing antibody titers.

The GMT neutralizing antibody titers in the group that received 50 μg of vaccine with MF59 were greater than the titers in recipients of 50 μg of vaccine without adjuvant, but this difference was not statistically significant ($P = .08$) (Table 4). However, the neutralizing antibody titers achieved in groups receiving VLPs with MF59 or with no adjuvant were significantly higher than the titers in the group receiving VLPs in alum ($P = .0001$ and $P = .001$, respectively). Thus, the relative hierarchy of the GMT neutralization resembled the GMT ELISA titers.

Discussion

The results presented here indicate that three intramuscular doses of 10 or 50 μg of HPV16 L1 VLP vaccine with no adjuvant, with alum, or with MF59 were well tolerated and highly immunogenic in normal human volunteers. Regardless of dose and whether or not adjuvant was present in the vaccine, each of the volunteers who received the vaccine demonstrated a serum immune response by 1 month after the second immunization (i.e., at month 2). The third immunization induced a further elevation in end point serum antibody titers in most instances. These results complement, for a high-risk HPV type, a recent report of HPV VLP vaccination in a therapeutic setting *(31).* In that study, multiple injections of 1, 5, or 10 μg of HPV6 VLPs, without adjuvant, were well tolerated in genital wart patients with HPV6 infections, and vaccination induced an increase in VLP ELISA titers in most patients.

The most commonly reported side effect in our study was pain at the site of injection. Most of the pain was mild and short-lived, consistent with other intramuscularly administered recombinant subunit vaccines, such as licensed hepatitis B vaccines *(32,33).* Side effects were similar in recipients of placebo and recipients of 10 μg of vaccine without adjuvant. However, recipients of the higher vaccine dose (50 μg) without adjuvant did report more side effects than the placebo recipients. As expected, a greater proportion of recipients of HPV16 L1 VLP with MF59 reported moderate pain at the injection site than recipients of HPV16 L1 VLP vaccine given alone or with alum. The proportion of subjects experiencing injection site reactogenicity after injection of HPV16 L1 VLP with MF59 was comparable to that reported with other investigational vaccine antigens given with MF59, such as hepatitis B surface antigen and herpes simplex type II gD glycoprotein *(34,35).*

In this study, the adjuvant effects of alum and MF59 were evident in recipients of the lower vaccine dose (10 μg). In addition, there was a dose-dependent response in the groups receiving vaccine without adjuvant or with MF59, although not in the groups receiving vaccine with alum. Specifically, as determined by GMT ELISA titers, there was a marked increase in the immune response to 50 μg without adjuvant compared with 10 μg without adjuvant (10 240 versus 640), a modest increase with MF59 (10 240 versus 3480), and no increase in the ELISA titers seen with alum (3040 versus 2190). Since at the 50-μg dose the ELISA titers in recipients of vaccine without adjuvant were comparable to the titers in the group that received vaccine with MF59, and even higher than the group that received vaccine with alum, there was no apparent benefit to using adjuvant at this dose when serum antibody titers were measured shortly after immunizations. It is unknown whether these properties would continue to be true following a longer interval after the third vaccine dose. Since the addition of MF59 adjuvant was associated with increased pain and induration at the injection site, the optimal immunogenicity and reactogenicity profile in the current study was obtained with 50 μg of HPV16 L1 VLP vaccine without adjuvant. It is encouraging to note that, with the higher dose without adjuvant or with MF59, the final serum titers were about 40 times higher than those detected systemically after natural infection in the subjects who were seropositive before vaccination.

The similar titers seen at the 50-μg dose with no adjuvant or with MF59, combined with the modest increase seen with MF59 at the higher dose, suggest that even higher doses of vaccine would probably not induce substantially higher antibody titers. For alum, the 10-μg vaccine dose induced maximum ELISA titers. A similar plateau effect was reported when alum was used for HPV11 L1 VLP vaccination of macaques *(36).* Although we do not understand the basis for this phenomenon, two factors that can have a substantial impact on the immune response of antigens delivered with alum are the degree of antigen adsorption onto alum and the dose of alum used *(37).* It is unlikely that the degree of antigen adsorption adversely affected immunogenicity because, in the formulation containing 50 μg of HPV16 L1 VLP with alum, more than 95% of the VLPs was complexed with the aluminum hydroxide. However, even with high levels of antigen adsorption, it is possible that the concentration of alum was insufficient to induce a maximal adjuvant effect. It is also possible that the VLPs in alum, which were stored at 4 °C, might have been less stable. Such instability would presumably have affected only the 50-μg dose, which was given after the 10-μg dose.

A predominantly IgG1 response was also not unexpected. We have found recently that IgG1 is also the IgG isotype that predominates after seroconversion to natural infection *(25).* Predominantly IgG1 responses are also commonly seen after other microbial infections *(38).* In C57BL/6 mice, HPV16 L1 VLPs induce a more varied IgG response, with substantial amounts of specific IgG1, IgG2b, and IgG3 detected (Heather Greenstone, Ph.D. Thesis, The Johns Hopkins University). Whether this reflects a stronger Th1 type response to HPV VLPs in humans than in mice remains to be determined. The inability to detect an IgG2 response in most vaccine recipients is unlikely to be due to poor sensitivity or specificity of the IgG2 assay. In fact, the sensitivity/specificity of the IgG2 assay was superior to that of the IgG1 assay for isotype-specific human Ig control subjects *(25).*

Neutralization assays are often considered to be the "gold standard" in assessing the immunogenicity of a prophylactic vaccine such as the one tested here. When the month-5 sera from the groups that received the 50-μg dose of vaccine were analyzed for HPV16 peudovirion neutralizing activity, there was an excellent quantitative correlation with the ELISA titers. This correlation held for individuals as well as for groups, further implying that the ELISA appears to represent an appropriate surrogate assay for the more cumbersome and expensive neutralization assay.

Table 6. Animal studies demonstrating the ability of virus-like particles to protect against experimental papillomavirus challenge*

Investigator(s) (reference No.)	Model	Dose—adjuvant	Vaccination schedule, wk	ELISA titer	Time†
Breitburd et al. *(14)*	CRPV—rabbit	3 × 50 μg—alum	0, 2, 4	5000	+1 wk
Kirnbauer et al. *(17)*	BPV4—cow	2 × 150 μg—alum	0 and 4	1000	+2 wk
Suzich et al. *(16)*	COPV—dog	2 × 20 μg—none	0 and 2	<1000	+2 wk
Christensen et al. *(15)*	CRPV—rabbit	3 × 50 μg—none	0, 4, and 8	10 000	+2 wk
				100	+12 mo

*ELISA = enzyme-linked immunosorbent assay; CRPV = cottontail rabbit papillomavirus; BPV4 = bovine papillomavirus type 4; COPV = canine oral papillomavirus.

†Time between last vaccination and virus challenge.

For both assays, there was a remarkable consistency of response within each vaccine group. The immunogenicity results seen here with the human volunteers parallel those obtained in animals, where consistently statistically significant immunogenicity and protection against experimental disease have been observed, even when adjuvant was not given. It is likely that the particulate nature and regular array of L1 in the VLPs contribute to their high immunogenicity. Perhaps efficient immune recognition is promoted by an interaction between the VLPs and cell surface pattern recognition receptors that bind the ordered external structure of icosahedral virions *(39)*.

It is not possible to know from these studies whether systemic administration of vaccine will protect against cervical infection under natural conditions. However, the magnitude of the antibody responses in the human volunteers compares favorably with that seen in animal studies in which VLP vaccination, with alum or without adjuvant, induced protection from high-dose virus challenge (Table 6). Although some caution must be taken in comparing studies in which different vaccination protocols and ELISAs were used, our overall impression is that humans and experimental animals respond similarly to VLP vaccination.

The animal models used experimental inoculation of abraded epithelium and tested cutaneous and oral mucosal infection, rather than genital infection. However, there are reasons to suspect that anticapsid antibodies generated systemically could at least partially neutralize natural HPV16 infection, even though HPV infection and replication occur in the epidermis of the genital tract. First, it is likely that HPV infection of the basal cell layer requires microtrauma or abrasion of the epidermis *(4)*. Such disruption of the epidermis could expose the virus to serous exudate containing neutralizing IgG antibodies. Second, in immune-mediated dermatologic diseases, such as pemphigus vulgaris, circulating antiepidermal antibody traverses the dermal–epidermal junction, suggesting that the basement membrane is not an impermeable barrier to immunoglobulins *(40)*. Third, statistically significant levels of IgG have been reported in cervicovaginal secretions. Much of this IgG is likely the result of transudation from the circulation *(41)*. Lowe et al. *(36)* demonstrated in African green monkeys that parenteral immunization with as little as 10 μg of HPV11 L1 VLPs induced detectable IgG antibody titers in cervicovaginal secretions. Although lower in magnitude, these titers closely paralleled the serum responses. In addition, the IgG present in the cervicovaginal secretions was sufficient to neutralize HPV11 virus. It is, therefore, plausible that parenteral immunization with HPV16 L1 VLPs could result in the appearance of HPV16-specific IgG in cervicovaginal secretions in humans. Even if VLP vaccination did not achieve sterilizing immunity, it might, nevertheless, substantially modify the incidence and duration of HPV16-induced genital neoplasia by restricting virus replication and the extent of primary infection. It is likely that a reduction in viral load would also diminish transmission to sex partners.

The safety and immunogenicity profile obtained in this study encourages further clinical investigation of HPV VLP-based immunoprophylactic vaccines. On the basis of these results, we have initiated a phase II trial of 50 μg of L1 VLP without adjuvant. The key question of whether systemic administration with a VLP vaccine can confer protection under natural conditions must await the outcome of controlled efficacy trials. If the vaccine were eventually shown to be effective, the type specificity of the neutralizing activity induced by the VLPs implies that protection would be type specific. Since multiple HPV types are implicated in cervical cancer, a multivalent vaccine would be needed. A vaccine composed of the four HPV types seen most frequently in cervical cancer (types 16, 18, 31, and 45) would theoretically be able to protect against approximately 80% of cervical cancers *(6)*.

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NOTES

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